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Comparative Biochemistry of Hemoglobins

II. Various Kinds of Components in Newborn Bovine Hemoglobin and Their Quantitative Changes after Birth

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In the course of the study on the hemoglobin contained in newborn bovine blood, two major and five minor components which are specific to newborn bovine hemoglobin were detected by the methods of agar-gel electrophoresis, cellulose-acetate membrane electrophoresis and column chromatography. These components were examined on their alkaline resistance, and the results showed that all the components of newborn bovine hemoglobin were less resistant than those of adult bovine hemoglobin, and that these components are different in their alkaline resistance one another. In addition, the quantitative changes of the components in newborn bovine hemoglobin accompanying the development after birth were investigated.

INTRODUCTION

The comparative studies on hemoglobins have been made in this laboratory[7] for these several years. Connecting with this problem, the author has studied various components of newborn bovine hemoglobin. It is well-known that many mammalians have a different type of hemoglobin at their fetal stage from the mature type. In the case of human hemoglobin, for example, fetal human hemoglobin, which has played the main part of respiration in the fetus, gradually decreases after birth, and it almost completely disappears in four months after birth, being replaced by adult human hemoglobin. Moreover, a clear structural difference is observed between this fetal hemoglobin and the adult one. This phenomenon is significant because it stands for the transition of ability of protein biosynthesis in the development of living matter.

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However, we find various difficulties in making experiments on the control mechanism of hemoglobin biosynthesis in the development using human hemoglobin. Therefore, it is more convenient to study this control mechanism on fetal or newborn hemoglobin of other mammalians.

Fetal or newborn bovine hemoglobin was first suggested by BRINKMAN and JONXIS[2] to be different from adult bovine hemoglobin using the examination on their resistance to alkali. Then, WYMAN *et al.*[11] also recognized the difference between fetal bovine hemoglobin and the adult one because the former has a higher solubility than the latter. Later, CABANNES and SERAIN[3], BARAK[1], and MONNIER and FISCHER[9] reported that fetal bovine hemoglobin was distinguishable from the adult one by electrophoresis. Moreover, HUISMAN *et al.*[5] stated that one major and two minor components, which were considered to be peculiar to the fetal bovine, could be identified by column chromatography. The present author isolated hemoglobin from the blood samples obtained from newborn bovines of the Holstein type at various stages of development ranging from 6 hr to 35 days after birth, and studied on various kinds of components contained in the hemoglobin samples using agar-gel electrophoresis, cellulose-acetate membrane electrophoresis, column chromatography, and the alkaline denaturation method.

MATERIALS AND METHODS

1) Preparation of Hemoglobin Solution.

Five cases of newborn bovines of the Holstein type (cases 1, 2, 3, 4 and 5) and one case of an adult bovine (case 6) were investigated. The samples were obtained by drawing venous blood from the jugular vein 6 hr, 2 days, 15 days, 25 days and 35 days after birth in case 1; 3 days, 12 days, 22 days, 32 days after birth in case 2 ; 3 days, 13 days, 23 days, 33 days after birth in case 3 ; 2 days after birth in case 4 ; 7 days after birth in case 5 ; and at the adult stage in case 6. The preparation of the hemoglobin solution was carried out according to DRABKIN's method[4]. The procedure, briefly describing, is this. Red blood cells are isolated by centrifuging the blood sample at 1,000 – 2,000 r. p. m. for 5 min at 0°—5°C after the addition of sodium citrate to prevent blood coagulation, and washed three times with 0.9% NaCl solution to remove the plasma. The washed red blood cells are hemolyzed by adding 2 volumes of distilled water and 0.4 volume of toluene, and the hemolysate is separated into three layers when it is centrifuged at 15,000 r. p. m. for 60 min at 0°—5°C. The middle layer is the desired hemoglobin solution.

2) Agar-gel Electrophoresis

The agar-gel electrophoresis of the hemoglobin solution was carried

out according to SHIBATA's method[10]. Using Tris-EDTA-borate buffers, pH 8.6 (15.0 g of Tris-(hydroxymethyl) aminomethane, 2.3 g of ethylenediamine-tetraacetic acid, and 3.0 g of boric acid per 1 L) and pH 7.2 (12.3 g of Tris-(hydroxymethyl)aminomethane, 4.8 g of ethylenediamine-tetraacetic acid, and 6.7 g of boric acid per 1 L), electrophoresis was performed at 0°—5°C, at 200 V for 60 min at pH 8.6, and at 100 V for 80 min at pH 7.2.

3) Cellulose-acetate membrane Electrophoresis

The procedure of cellulose-acetate membrane electrophoresis of the hemoglobin solutions was that described by KOHN[6] with a little modification. The cellulose-acetate membrane employed was Oxoid (manufactured at Oxo Co., England) and was cut into 1 × 5-cm strips. Using Tris-EDTA-borate buffer, pH 8.0 (6.05 g of Tris-(hydroxymethyl)aminomethane, 6.00 g of ethylenediamine-tetraacetate 2 Na, 4.60 g of boric acid per 1 L), electrophoresis was carried out at 0.4 mA/cm for 60 min at room temperature, and the hemoglobin was stained with Ponceau 3 R.

4) Column Chromatography

Using CM-cellulose for the adsorbent and phosphate buffer for the developer, column chromatography was performed by the gradient elution in pH. First, for activation, CM-cellulose (0.7 meq/g, manufactured at Serva Co., Heidelberg) was washed thoroughly with acetone, distilled water, 1 N ammonia, distilled water, 1 N hydrochloric acid, and distilled water successively. After washing again with 0.01 M sodium phosphate buffer, pH 6.4 (containing 0.01% KCN) thoroughly, the CM-cellulose was packed into a 1 × 60-cm chromatographic column up to the height of 50 cm and equilibrated. Against 500 cc of this buffer, a 2cc sample of the hemoglobin solution containing about 100 mg of hemoglobin was dialyzed for a whole day and night, and placed on the column. Then with 250 cc of the above-mentioned buffer in the mixing bottle and 250cc of 0.01 M sodium phosphate buffer, pH 7.0 (containing 0.01 % KCN) in the supplying bottle, gradient elution was performed at the flow rate of 0.5 cc/min. When the buffer in the supplying bottle had flowed off, elution was continued by adding in it 500 cc of 0.01 M sodium phosphate buffer, pH 8.5 (containing 0.01 % KCN). The effluent from the column was collected in 5cc fractions, and the absorbance at 415 mμ was measured by a Spectronic 20 (Bausch and Lomb Co.), and the pH by a pH meter.

5) Alkaline Denaturation Method

Every component separated by CM-cellulose column chromatography was subjected to the alkaline denaturation test according to the method which was described before[8]. The hemoglobin solution was diluted so as to be near to 0.7 in the absorbance at 415 mμ using a Spectronic

20. The absorbance of the mixture of 3 cc of this solution and 1 cc of distilled water was measured and the value was termed E_0. Subsequently, 3 cc of hemoglobin solution and 1 cc of phosphate buffer pH 12.9 (containing 65.3 g of $Na_2HPO_4 \cdot 12H_2O$ per 400 cc, adjusted to pH 12.9 with 1 N NaOH) were mixed and the absorbance of the mixture was measured every minute up to 15 min and termed E. Then, this solution was placed in a water bath at 37°C for 15 min and the absorbance of the solution at this time was measured and termed Ee. Thus, the percentage (P) of the denatured hemoglobin was obtained from the following equation:

$$P = \frac{E_0 - E}{E_0 - E_e} \times 100$$

RESULTS AND DISCUSSION

Fig. 1 shows the results of agar-gel electrophoresis of newborn bovine hemoglobin (cases 1 and 2) and adult bovine hemoglobin (case 6). At pH 8.6, two major components peculiar to newborn bovine hemoglobin can be identified in blood samples obtained 6 hr and 2 days after birth in case 1, and 3 days and 12 days after birth in case 2. At pH 7.2 also, there is a distinguished difference between newborn hemoglobin and the adult one, but separation between the two major components peculiar to the newborn bovine is not so satisfactory.

Fig. 2 shows the results of the cellulose-acetate membrane electrophoresis. For samples, in addition to the cases 1 and 2 used for agar-gel electrophoresis, case 3 was employed. In every case of cellulose-acetate electrophoresis, the two major components peculiar to the newborn bovine can be more clearly recognized.

Fig. 3 gives the column chromatogram of the hemoglobin isolated from the blood sample obtained 6 hr after birth in case 1. Besides the two major components (F_0^A and F_0^B), which were recognized to be peculiar to the newborn bovine in the above-mentioned electrophoretic experiments, some minor components, F_I^A, F_I^B, F_I^C, F_{II} and F_{III} were found. In the present experiment of column chromatography, F_I^A, F_I^B and F_I^C were eluted before F_0^A, that is, below pH 6.6. F_0^A was observed to be eluted at about pH 6.7; F_{II} at about pH 6.8, between F_0^A and F_0^B; F_0^B at about pH 7.6; a major component A_0, which was due to adult bovine hemoglobin, and minor components A_I^A and A_I^B between pH 7.1 and 7.5; and the last F_{III} at about pH 7.7. While HUISMAN *et al.* [5]. already reported that they had found one major and three minor components specific to fetal bovine hemoglobin by column chromatography, it is of interest that in the present work two major and five minor components have been observed.

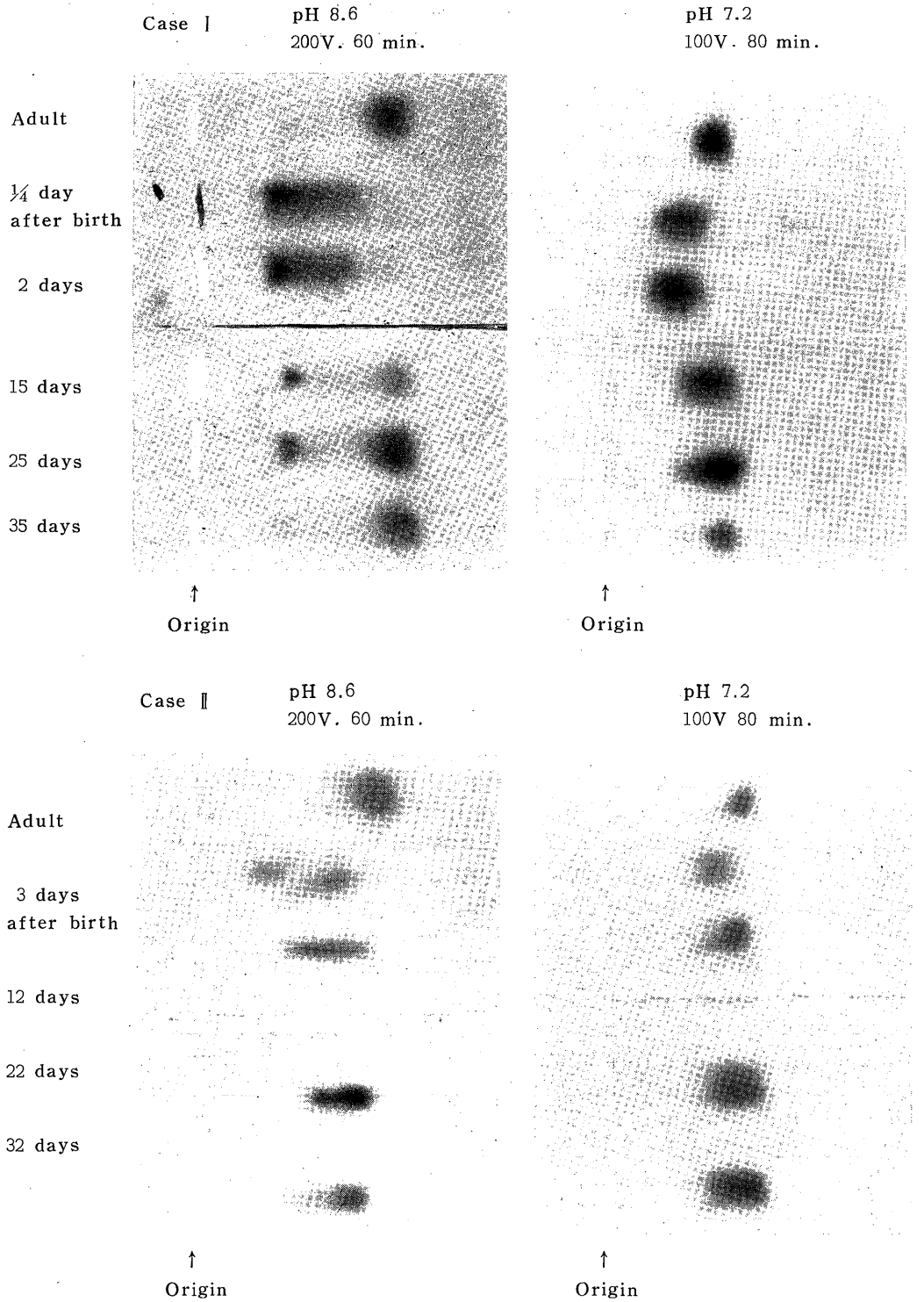

Fig. 1. Agar-Gel Electrophoresis of Newborn and Adult Bovine Hemoglobins

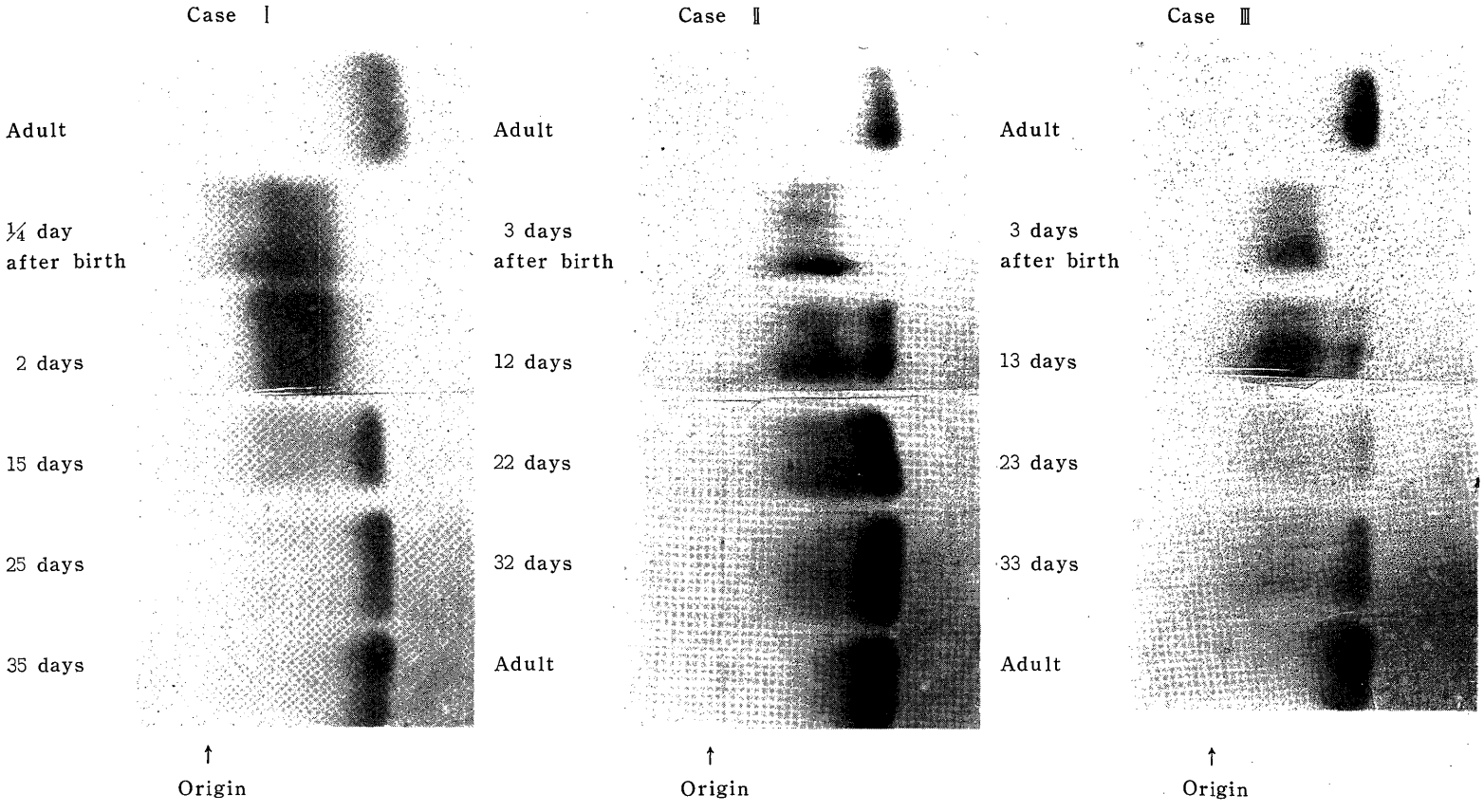

Fig. 2. Cellulose-Acetate Membrane Electrophoresis of Newborn and Adult Bovine Hemoglobins. Tris-EDTA-Borate Buffer (pH 8.0), 0.4 mA/cm. 60 min.

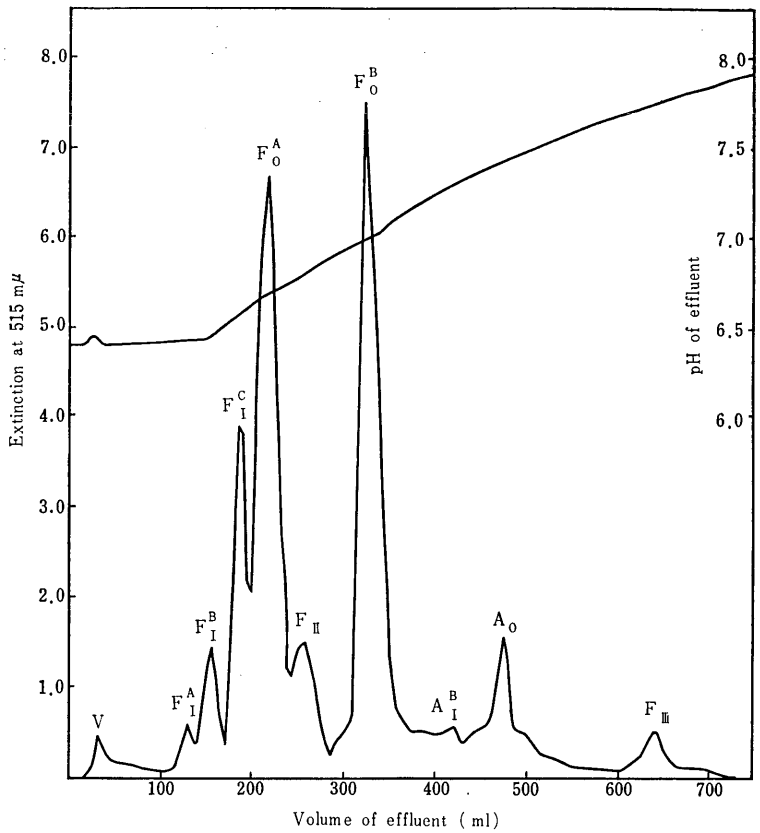

Fig. 3. Chromatogram of Newborn Bovine Hemoglobin.
(case 1. 1/4 day after birth.)

Fig. 4 represents the hemoglobin in the blood sample obtained 2 days after birth, giving almost the same results as that obtained 6 hr after birth.

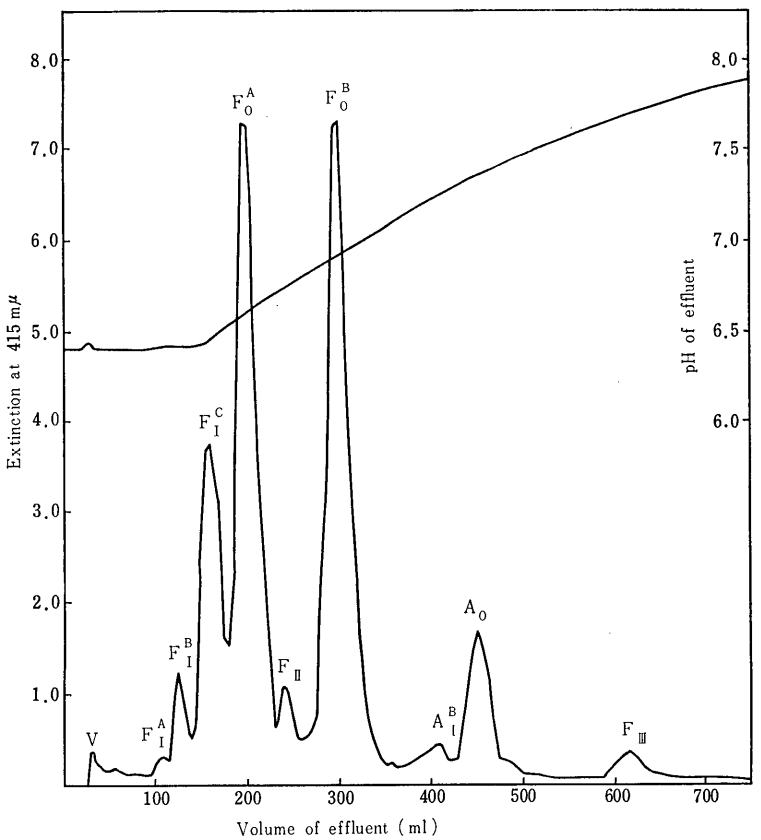

Fig. 4. Chromatogram of Newborn Bovine Hemoglobin.
(case 1. 2 days after birth.)

Fig. 5 shows the hemoglobin in the blood sample obtained 15 days after birth, giving a marked decrease of the F group and a remarkable increase of the A group.

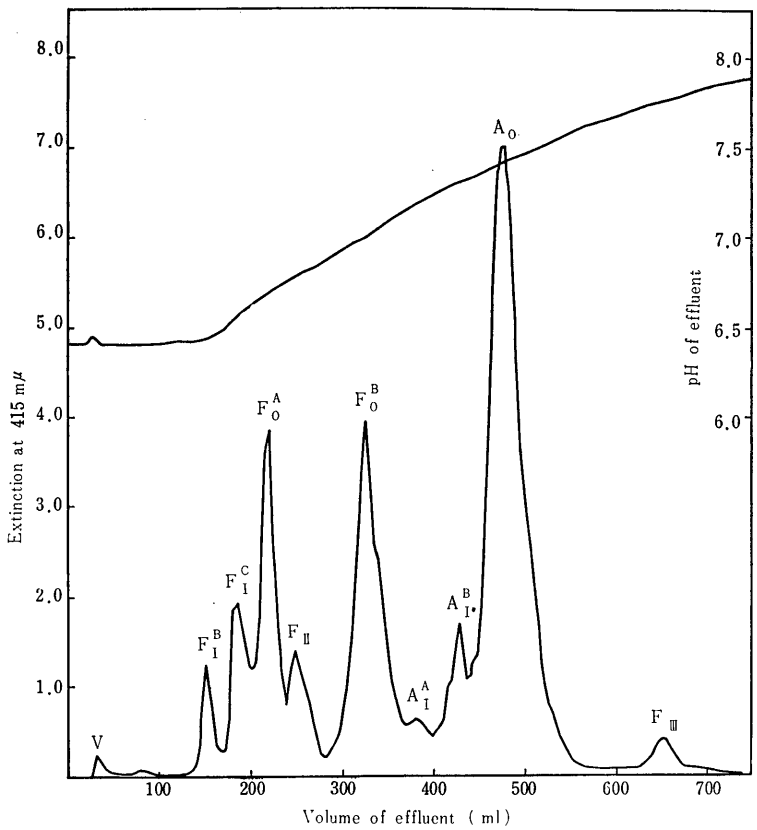

Fig. 5. Chromatogram of Newborn Bovine Hemoglobin.
(case 1. 15 days after birth.)

Fig. 6 represents the hemoglobin in the blood sample obtained 35 days after birth, in which the F group keeps only its trace, F_I^A and F_{III} having already disappeared, and the A group is predominant.

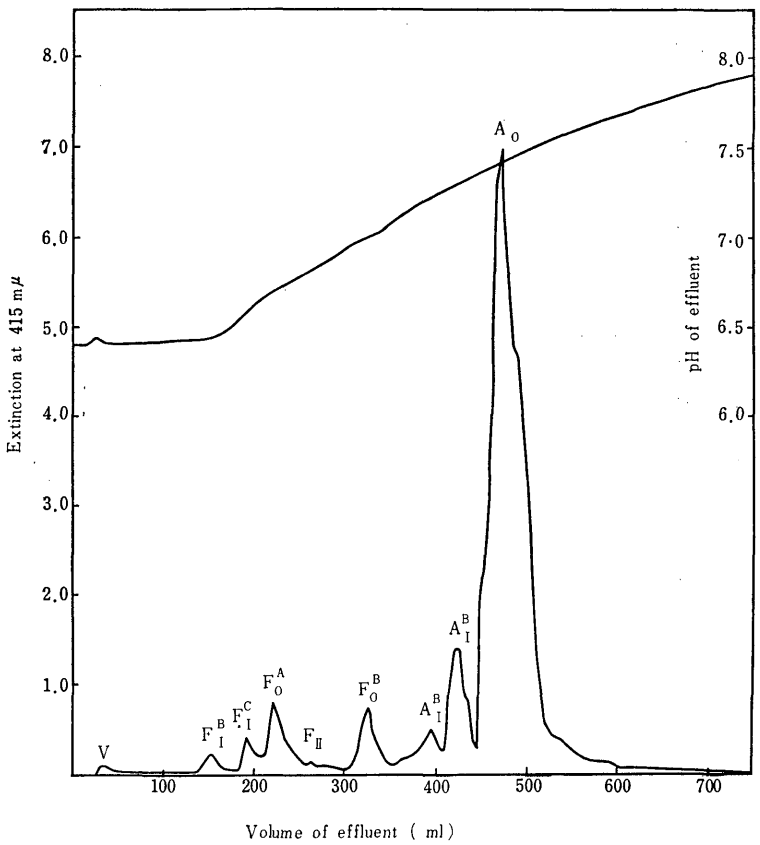

Fig. 6. Chromatogram of Newborn Bovine Hemoglobin.
(case 1. 35 days after birth.)

In Fig. 7, the column chromatogram of adult bovine hemolobin is shown for comparison, which consists of one major and two minor components as HUISMAN *et al.*[5] have reported.

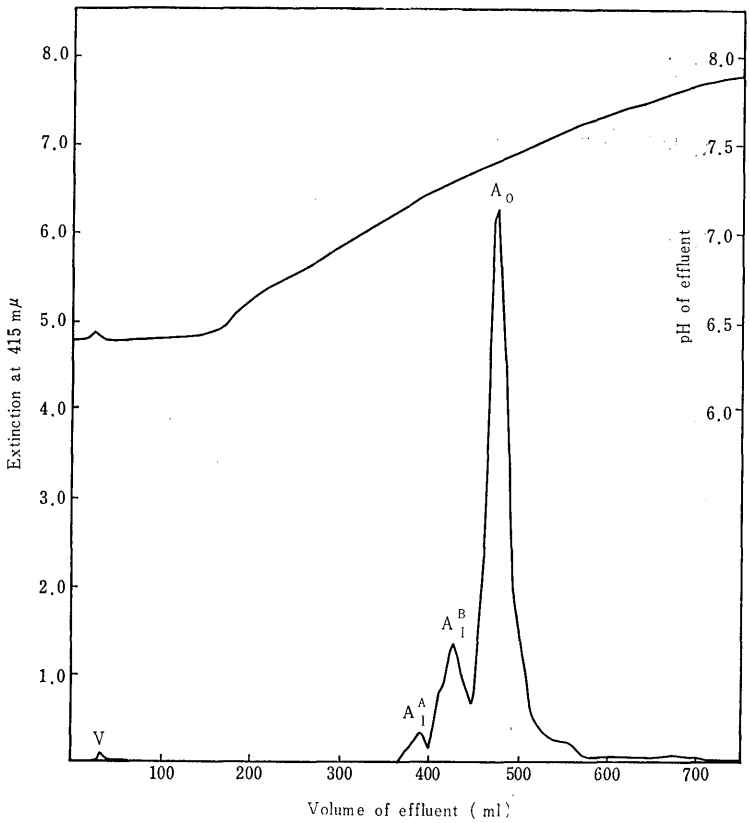

Fig. 7. Chromatogram of Adult Bovine Hemoglobin. (case 6)

Fig. 8 illustrates the resistance to alkali of the various components separated by the above-mentioned column chromatography. There, all the components specific to newborn bovine hemoglobin are less resistant to alkali than the components of adult bovine hemoglobin. Among them, F_0^B is especially less resistant, F_0^A, F_I^A, F_I^C and F_{III} have almost similar resistance to alkali, intervening between F_0^B and the group of the adult bovine hemoglobin components, namely, A_o, A_I^A and A_I^B, and F_{II} is nearest to the group of A_o, A_I^A and A_I^B. These results are quite opposite to the relationship between human fetal and human adult hemoglobins.

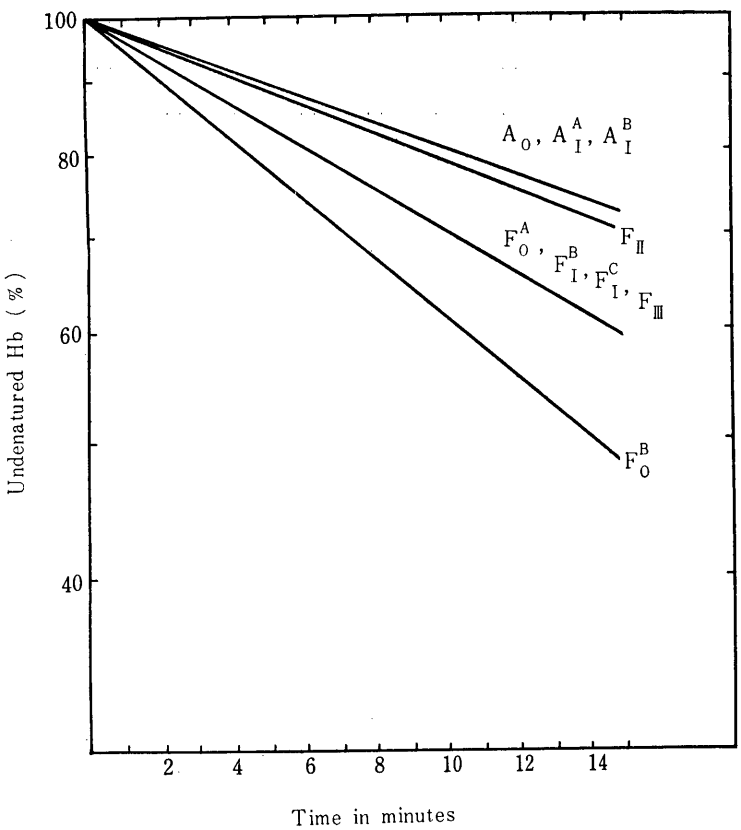

Fig. 8. Alkaline Denaturation of Each Component from Newborn and Adult Bovine Hemoglobins.

Table 1.
Percentages of different hemoglobin components in blood samples of newborn and adult bovines

Case	Days after birth	Hemoglobin components										
		V	F_1^A	F_1^B	F_1^C	F_0^A	F_{11}	F_0^B	A_1^A	A_1^B	Ao	F_{111}
1	¼	1.05	1.89	4.65	13.29	30.37	6.49	30.37	—	2.00	7.20	2.69
	2	0.87	0.88	3.15	15.86	30.68	3.27	33.44	—	1.81	8.17	1.85
	15	0.46	—	2.91	5.59	12.47	4.85	18.57	2.06	6.29	44.98	1.82
	35	0.41	—	1.03	2.04	4.66	0.58	3.79	3.61	8.39	75.49	—
4	2	1.00	—	3.90	10.13	30.72	3.23	37.09	—	3.08	8.00	2.84
5	7	1.11	—	4.68	9.74	24.94	4.05	35.83	—	1.98	15.63	2.05
6	Adult	0.28	—	—	—	—	—	—	2.24	16.91	80.57	—

Table 1 summarizes the quantitative changes in the content of the various components of newborn and adult bovine hemoglobins examined by the above-mentioned column chromatography relating to the number of the days after birth. From the birth to one week after, the two major components of newborn bovine hemoglobin, F_0^A and F_0^B, are observed to be about 30 % respectively, whereas the main component of adult bovine hemoglobin, A_0 is found to be no more than about 8% at first and 15 % one week after. However, 35 days after birth, F_0^A and F_0^B decrease down to 3 – 5 %, whereas A_0 increases up to about 75 %. And after that, all the components specific to the newborn bovine, including F_0^A and F_0^B, disappear almost completely and only the group of the components specific to the adult bovine, namely, A_0, A_1^A, and A_1^B can be found. From the above-mentioned experimental results, it is considered that the Holstein type bovine has at least two kinds of major hemoglobin components and five minor hemoglobin components in its fetal stage, and that all these components of fetal hemoglobin are rapidly replaced by those of adult hemoglobin after birth, having almost completely disappeared in 35 days. The exchange is made more rapidly than the exchange from human fetal hemoglobin to adult hemoglobin. Therefore, the bovine hemoglobin is considered to be a more suitable material for the experiments to study the transition of ability of hemoglobin biosynthesis accompanying development of living matter.

CONCLUSION

Hemoglobins were isolated from newborn bovine blood at various stages ranging from 6 hr to 35 days after birth, and the components of these hemoglobins were investigated.

1. In the experiments by methods of agar-gel and cellulose-acetate membrane electrophoresis, there were observed two major hemoglobin components which were considered to be specific to fetal and newborn bovine hemoglobin.

2. By column chromatography using CM-cellulose, there could be separated two major and five minor components which were considered to be specific to fetal and newborn bovine hemoglobin. Then the quantitative changes of these components accompanying to development after birth were also examined by column chromatography.

3. Alkaline resistance was examined on these components separated by the column chromatography. All the hemoglobin components which are considered to be specific to fetal and newborn hemoglobin were less resistant to alkali than those of the adult hemoglobin components. Moreover, it was found that these hemoglobin components had different alkaline resistance one another.

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